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Long-term shelf-life liposomes for delivery of prednisolone and budesonide

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ABSTRACT

Liposomes are nanoscale drug delivery systems built up from lipid layers and are able to spontaneously self-assemble in an aqueous environment. Both hydrophilic and hydrophobic drugs can be delivered by liposomes and this kind of nanoformulation offers many advantages regarding biodistribution, drug absorption and controlled drug release. Corticosteroids as lipophilic molecules are able to integrate into the lipid bilayer. This novel approach can improve the efficacy of several anti-inflammatory, such as asthma therapy.

Our aim was to create liposomes with long shelf-life, which can incorporate and release corticosteroids such as Prednisolone (Pred) and Budesonide (Bud) at the temperature of inflamed tissues. Two kinds of liposome samples were prepared from three different kinds of phospholipids to get unilamellar vesicles with 100 nm in diameter and characterize their physicochemical properties and effect on living cells. Their main phase transition temperature in the physiologically relevant temperature range was measured by differential scanning calorimetry. According to the size distributions determined by dynamic light scattering, all drug-containing liposomes were stable for 6 months. All of the liposome types have a slightly negative zeta potential value. The Fourier-transform infrared spectroscopy revealed no chemical interaction between the drug and lipid molecules. The entrapment efficacy was determined by size-exclusion gel chromatography combined with UV–VIS spectrophotometry and it was very high in both cases (between 70 and 87%). The drug leakage was 35–40% for Pred and 6–8% for Bud in the first 30 min. The effect of liposomal drugs on cell viability was measured on the EBC-1 human lung carcinoma cell line. Neither the free corticosteroids nor their liposomal form were toxic to the cells. The cellular internalization of the liposomes was proved by flow cytometry and confocal microscopy.

In summary, these liposomes could be useful in the delivery of corticosteroids (Pred or particularly Bud) in more effective asthma therapy, having fewer side effects due to the nanoformulation.

1. Introduction

During the past two decades, nanomedicine has become an increasingly realistic alternative to conventional pharmacotherapy [1]. The solutions provided by nanomedicine mostly include new ways of delivering already existing drugs. However, nanoformulation of drug molecules leads to unique properties and is accompanied by advantages in both physicochemical (like the controlled release, increased solubility, and high specific surface area) and biological (bioavailability, biocompatibility, and lower risk of the immune response) points of view [1,2]. Recently, a wide variety of nanomaterials has been developed for drug delivery, including metallic nanoparticles, carbon-based nanostructures, protein or polymer formulations and liposomes [1–3].

Lipid-based nanoparticles have significant importance in the field of

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medicine, especially since the COVID-19 pandemic where lipid nanoparticles were successfully used for formulation of mRNA vaccines [4]. Among the nanomedicines, liposomes resembling cell membranes are another type of promising drug carrier systems [1,3]. These vesicles composed of phospholipid bilayer(s) can be relatively easily fabricated and modified, as well as they are suitable for the delivery of practically any kind of biomolecules or drug molecules [2]. Phospholipids are amphiphilic molecules as they have both a hydrophilic (a water-soluble head containing phosphate group) and a lipophilic part (hydrophobic tail composed of long oil-soluble hydrocarbon chain(s)). In an aqueous environment, the phospholipid molecules are able to spontaneously self-assemble creating various structures, such as planar lipid bilayers, micelles or vesicles. In the 1970s, Gregoriadis discovered that the liposomes were able to entrap pharmacologically active molecules and he suggested their use as drug carriers [5]. After this, the field of liposomology developed rapidly [6,7].

The diameter of the liposomes ranges from tens of nanometers to several tens of micrometers, with the thickness of the bilayer being around 4 nm [8]. Liposomes can be classified according to their size and number of lipid bilayers. For drug delivery purposes, usually, unilamellar liposomes (composed of a single lipid bilayer) with a diameter range between 50 and 150 nm are the most suitable [7,8]. Considering an inhalation therapy for airway diseases (which is the focus of our research), particles with the size of 50–200 nm can accumulate deep in the lungs while particles over 200 nm in size are generally exhaled [2]. Once liposomes arrive at their target cells, they deposit their contents by adsorption, endocytosis, lipid exchange or membrane fusion [7].

Phospholipid bilayers can exist in two states: they are either in a fluid (liquid-crystalline) state or in a gel state. If the temperature is higher than the so-called main transition temperature (T_m), the lipid bilayer melts and transforms from the gel state to the liquid-crystalline state, providing higher permeability for the encapsulated drugs [9]. The transition temperature is highly dependent on the length and degree of saturation of the acyl chains composing the hydrophobic tail of the phospholipid [10]. Consequently, by applying lipid combinations, the T_m can be tailored and set for typical temperatures occurring in human medicine.

A great advantage of liposomes is that they can defend the delivered active substance from degradation by shielding the entrapped drug from the outside environment. Moreover, exposure of sensitive tissues to toxic drugs can also be decreased by using liposomes [7]. In spite of their favourable features, merely a dozen liposomal drugs have been approved by the FDA so far. However, none of them is indicated for asthma therapy [11]. Therefore, our aim was to develop a liposomal preparation for this therapeutic purpose.

Asthma is a chronic inflammatory disease of the airways [12] affecting more than 300 million people worldwide [13]. Asthmatic patients suffer from breathing difficulties, which can even lead to death in severe cases. Currently, there is no specific treatment for asthma but corticosteroids are often used in the therapy [13]. Natural corticosteroids are steroid hormones produced by the adrenal cortex and they are involved in several cellular functions including metabolism and inflammation [14]. Synthetic corticosteroids (such as prednisolone or budesonide) can successfully alleviate asthmatic symptoms due to their anti-inflammatory effect [13]. Nevertheless, their long-term and high-dose application is accompanied by severe side effects [14]. Nanoformulation of these drugs could enhance the therapeutic efficacy and reduce the side effects [13].

Although in vivo experiments [15,16] proved that liposomal budesonide can effectively mitigate inflammation in asthma, these studies lack the physicochemical characterization of the liposomes. In addition, research papers about the development of liposome preparation for asthma therapy [17–19] mostly describe a hardly standardizable production method or do not investigate the interactions of the liposomes with cells. Moreover, budesonide may have a remarkable role in the future during the battle against COVID-19 since its antiviral activity against SARS-CoV-2 was proved by *in vitro* tests [20] whereas a clinical trial demonstrated that inhalation of budesonide in the early phase of infection can reduce the risk of hospitalization [21].

The goal of this study was to create long-term shelf-life liposomes loaded with prednisolone and budesonide for possible future application in asthma therapy and to characterize both the physicochemical properties and the effect of these liposome preparations on living cells. Our aim was to compare liposomes with different lipid composition at a certain drug content concerning stability, entrapment efficacy and bioavailability. As the main lipid component, DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine) was chosen, which composes 31% of the lipid content of the pulmonary surfactant in lung alveoli [22]. In order to obtain high drug release at the temperature of inflamed tissues, the theoretical T_m of the liposomes was set to 39 °C by choosing appropriate amounts of other phospholipids. Moreover, we intended to investigate the effect of our liposomal preparations on the EBC-1 human lung carcinoma cell line and the internalization of the liposomes by the cells.

2. Materials and methods

2.1. Composition of the liposomes

Drug-free and either prednisolone or budesonide-containing liposomes were created utilizing phospholipids with saturated long-chain fatty acids (containing 14, 16, or 18 carbon atoms, respectively). Two types of liposomes were prepared: type 1 was composed of 2 lipid components (2C), namely 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC or 16:0 PC, Sigma-Aldrich, USA) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC or 14:0 PC, Sigma-Aldrich, USA) while the 3-component liposomes (3C) were composed of DPPC, DMPC and 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC or 18:0 PC, Sigma-Aldrich, USA). The chemical structures of the applied lipids and drugs can be seen in Fig. S1-.

We created liposomes with phase transition temperatures ($T_{physiol}$) in the physiologically relevant range close to the typical temperature of the inflamed tissues in the human body (around 39 °C). After preliminary calculations and experiments, the following lipid compositions were chosen: the molar ratio of DPPC and DMPC was 15:2 regarding the 2C liposomes, whereas a 7:4:3 M ratio was applied for DPPC, DMPC, and DSPC in the case of 3C liposomes. In the case of both types of liposomes, both corticosteroid drugs i.e. prednisolone (Pred, (≥99%, Sigma-Aldrich, USA) or budesonide (Bud, (≥99%, Sigma-Aldrich, USA) were added to the lipids at 10 (w/w) %, according to previous publications [23,24]. This 1:10 drug to lipid weight ratio corresponds to 1:5 M in case of Pred while 1:5.9 molar ratio regarding Bud. The precise compositions concerning the different types of liposomes can be found in Table 1.

Chemical structures of the applied corticosteroid drugs: prednisolone and budesonide can be seen in Fig. 1.

The T_m of neat lipids are known whereas the T_m of lipid mixtures can be calculated from the T_m values of the single lipid components based on their molar ratio. The theoretic T_m values of the applied 3 kinds of lipids as well as of 2C and 3C liposomes are summarized in Table 2.

Table 1
Compositions of the 2-component (2C) and 3-component (3C) liposomes loaded with either prednisolone (Pred) or budesonide (Bud), or without drug (w/o).

Sample name	Lipid components			Corticosteroids	
	DPPC	DMPC	DSPC	Prednisolone	Budesonide
2C w/o	8.9 mg	1.1 mg	–	–	–
2C + Pred	8.9 mg	1.1 mg	–	–	1.0 mg
2C + Bud	8.9 mg	1.1 mg	–	1.0 mg	–
3C w/o	5.0 mg	2.7 mg	2.3 mg	–	–
3C + Pred	5.0 mg	2.7 mg	2.3 mg	–	1.0 mg
3C + Bud	5.0 mg	2.7 mg	2.3 mg	1.0 mg	–

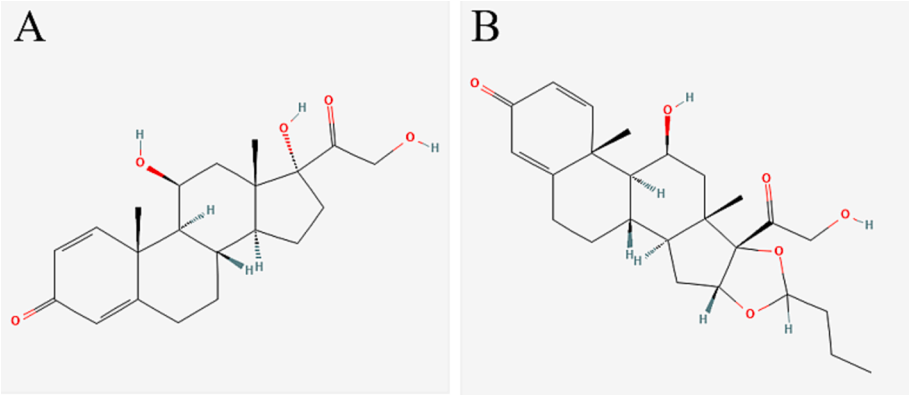

Fig. 1. Chemical structures of the applied drugs: prednisolone (A) and budesonide (B). *Source: PubChem database.*

Table 2
Known main phase transition temperatures (T_m) of the single lipid components [9] as well as the calculated T_m values regarding 2-component (2C) and 3-component (3C) liposomes without drug (w/o).

Sample	DPPC	DMPC	DSPC	2C w/o	3C w/o
T_m (°C)	41	24	55	39.0	39.1

2.2. Preparation steps of liposomes

2.2.1. Thin layer hydration

The liposomes were prepared by applying the conventional thin lipid layer hydration method followed by extrusion. First, the appropriate quantities of lipids (and the corticosteroid, if any) were dissolved in 350 μl of chloroform (≥99 %, Sigma-Aldrich, USA). Afterwards, the organic solvent was evaporated by means of pure nitrogen gas during continuous rotation of the glass vial by hand to get a thin lipid film. Then the vials were placed into a desiccator for 12–24 h in order to remove any residual chloroform. Subsequently, 1 ml of phosphate-buffered saline (PBS) solution (pH 7.4, I = 150 mM, made with distilled water from PBS tablets, Sigma-Aldrich, USA) was added to the lipid layer in 100 μl batches in each case. The aqueous solution was heated above 50 °C using a Block-Therm heating module type 656 (MTA KUTESZ, Hungary) for 1 min and vortexed for 10 s between the PBS batches. At the end of this process, we gained a suspension of multilamellar liposomes with 10 mg/ml lipid content (1 %, w/w) in PBS.

2.2.2. Extrusion

In order to create unilamellar vesicles with an approximate diameter of 100 nm, the liposome suspensions were extruded through polycarbonate membranes by means of an Avanti Polar Mini Extruder (Avanti Polar Lipids, USA). The heating block of the extruder was left to warm up and kept at 45.0 °C, which is above the T_m regarding all of our liposome samples, providing ideal conditions for the deformation of the liposomes during extrusion. The device is supplied by two Hamilton syringes (1–1 ml), and between them, filter discs (Whatman 10 mm predrain disc with a diameter of 10 mm, Cytiva, USA) and polycarbonate membranes (Whatman Nucleopore Track-Etched membranes, Cytiva, USA) with pore sizes of 400 or 100 nm were applied, respectively. Firstly, a membrane with a 400 nm pore size was used, and the sample was slowly passed between the two opposing Hamilton syringes 11 times. After that, the sample was extruded through the membranes with a pore size of 100 nm 31 times. Consequently, we gained liposome suspensions in PBS having 1 (w/w) % lipid content and supposedly unilamellar vesicles (according to the literature [24,25] and our unpublished data) with diameters around 100 nm. After the extrusion, these liposome samples were stored in 1.5 ml Eppendorf tubes at room temperature until further investigations.

2.3. Differential scanning calorimetry

In order to determine the phase transition temperatures ($T_{physiol}$) in the physiologically relevant range, differential scanning calorimetry (DSC) was carried out on the 6 different liposome samples by applying a TA Instrument DSC Q2000 (TA Instruments, USA) device. Since the 1 (w/w) % lipid content of the original samples is not high enough for the DSC measurements, the suspensions were ultracentrifuged after extrusion. Regarding each sample, 1 ml suspension was centrifuged at 99 990 RCF for 60 min at 4 °C in an Optima XPN-100 ultracentrifuge (Becton-Dickinson, USA). After that, the pellets were resuspended in 100 μl PBS, respectively, hence 10-fold higher lipid content was achieved. From these liposome suspensions with 10 (w/w) % lipid content, 15–18 mg weighted samples were placed in T_{zero} Hermetic type aluminium sample holders (TA Instruments, USA), which were sealed by pressing. The thermal program included an equilibration at 20 °C followed by a heat ramp to 50 °C with a heating rate of 1 °C/min, after a long optimization process, considering the literature protocols [26] The sample holders were closed tightly, and besides, we used nitrogen as a purge gas at a rate of 50 ml/min. From the DSC spectra, we determined the peak temperature indicating the phase transition of the liposomes between 30 and 50 °C, according to the ISO 11357–3 international standard for Differential scanning calorimetry (DSC) by means of the TA Universal Analysis software (Fig. S2). Moreover, the T-on-set, T-off-set, and enthalpy (ΔH) values were also calculated. However, we could not prepare pure DSPC liposomes because of technical problems since for this type the extrusion temperature should be around 60 °C (at least by 5 °C above the theoretic T_m).

2.4. Dynamic light scattering (DLS)

To follow the potential changes in the size distribution of liposomes during 6-month-long storage in PBS, DLS measurements were carried out in the case of each sample on the day of the preparation, then on a weekly basis for the first month and monthly afterwards. At each time point, 20 μl of the suspension was diluted by adding 80 μl of PBS solution in an Omega cuvette (225288). Measurements were performed using an Anton Paar Litesizer 500-type particle size analyser (Anton-Paar, Austria) operating with a laser light of wavelength 658 nm, at a detection angle of 90°. For each liposome type, 3 parallel samples were prepared, and regarding each sample, the particle size distribution was determined from 3 series (60 measurements/series). For analysis of the data, the Kalliope software (version 2.20.0) was used. After determining the size distribution and the polydispersity, the liposome suspensions were kept at room temperature until further measurements.

Before the *in vitro* experiment, we investigated by DLS how the cell culture medium influences the average hydrodynamic diameter and polydispersity of the liposomes. In this case, 4 h long incubation in cell

culture medium was applied, similarly to the cell viability assay and study of internalization.

In order to examine the cellular uptake of the liposomes, they were labelled with the PKH67 fluorescent membrane dye (Sigma-Aldrich, USA). The procedure is described in 2.8. in detail. The effect of this fluorescent labelling on the size distribution of the liposomes was also studied by DLS.

The measurement of zeta potential was carried out by the same Anton Paar Litesizer 500 device which was used for determination of the size-distribution. Regarding each type of liposome suspensions, 70 μl were diluted with their solvent (PBS) to 700 μl and pipetted into an Omega Cuvette for zeta (No. 225288). In one run, 200 zeta potential measurements were evaluated and 3 parallel runs were made from each sample at room temperature. The data were analysed using the Kalliope software (version 2.20.0).

2.5. Attenuated total reflectance (ATR) Fourier-transform infrared spectroscopy (FTIR)

The ATR-FTIR spectra of the 5 different components in powder form (three phospholipids and two drugs separately) and the 6 types of liposomal suspensions were measured by Jasco FT/IR-4700A instrument equipped with a diamond ATR head (Jasco Ltd., ATR Pro ONE) in the range between 400 and 4000 cm^{-1} wavenumbers and at a resolution of 2 and 4 cm^{-1}. During each measurement, 64 scans were summarized. The background was air in both cases (powders and liposomes). In case of the powders (DPPC, DSPC, DMPC, Pred, and Bud), the measured amount was a few milligrams while 10 μl was applied from the liposomal suspensions (2C and 3C, without drug, with Pred or Bud) on the surface of the device.

2.6. Investigation of drug entrapment capacity and drug release

The entrapment efficiency was determined by size-exclusion gel chromatography applying qEVoriginal / 70 nm Gen 2 type columns (Izon Science Limited, New Zeeland). The schematic representation of this procedure is shown in Fig. S3. Three parallel samples were examined for each type of liposome and PBS buffer was used for elution. First, the columns were washed twice with 10 ml PBS then 0.5 ml of the freshly prepared suspensions of small unilamellar liposomes were pipetted on the top of the column. Fractions of 0.5 ml were collected and the absorbance of Pred (249 nm) and Bud (245 nm) was measured by Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). Since the drug-free liposomes also exhibit absorbance in this wavelength range, this was considered as background and used for correction of the values measured in case of the drug-containing liposomes. The full UV–Vis spectra of the 6 different kinds of liposomes without drug and with Pred or Bud can be found in Fig. S4. The drug concentrations in the fractions were calculated from the corrected absorbance values by means of calibration lines. The drug entrapment efficiency was determined considering the initial drug content (the weight of the drug added to the lipids during the liposome preparation) and the sum of the drug content of the obtained fractions containing only free drug molecules (excluded drug) and no liposomes, according to the following equation:

$$Entrapment\ efficiency\ (EE\%) = \frac{initial\ drug\ [mg] - excluded\ drug\ [mg]}{initial\ drug\ [mg]} \bullet 100\%$$

In order to check the supposedly high entrapment efficiency of the PKH67 fluorescent membrane dye, the PKH67-labelled liposomes were first separated from the supernatant containing the excluded (free) dye by means of the aforementioned size-exclusion gel chromatography. Then the amount of the excluded dye was determined in the liposome-free fractions by high-performance liquid chromatography applying an Agilent Series 1100 LC system (Agilent Technologies, USA). The analytes were separated on an Agilent Zorbax® Eclipse Plus C18 column (4.6 mm × 150 mm, 5 μm). The column and the autosampler were maintained at 25 °C. 10 μl of the sample was eluted under isocratic conditions over 3 min at a flow rate of 1.5 ml/min. The mobile phase was composed of the water-acetonitrile mixture (30:70, v/v). The detection was carried out by a UV-DAD detector at 260 nm and the eluted peaks were recorded at excitation and emission wavelengths of 490 and 502 nm, respectively with a PerkinElmer Series 200 fluorescence detector (PerkinElmer. MA, USA). The 18 fractions (1–1 ml) obtained by gel chromatography were measured to determine the amount of the excluded dye. Fractions of the pure dye stock solution were measured as a reference under the same conditions, thus establishing the recovery of the sample preparation. The exact amount of the dye was calculated according to a calibration curve. To quantify the amount of drug leakage from the liposomes, a dialysis setup was used. A cellulose dialysis membrane was filled with 500 μl of liposome suspension and placed into a beaker containing 40 ml PBS solution, a magnetic stirrer, and a mercury thermometer to monitor the temperature. For each type of liposome, dialysis was performed simultaneously at 37 °C, and 41 °C. At the higher set temperatures, evaporation of the liquid was observed, so the liquid level was checked periodically, and the beaker was refilled accordingly.

The amount of corticosteroids released from the liposomes was determined using an Agilent 8453 UV-VIS spectrophotometer (Agilent Technologies, USA). For this purpose, 2 ml of medium from outside of the dialysis membrane was transferred into a quartz cuvette at each experimental time point: every 10 min during the first hour, every 30 min between 1 and 3 h, and every hour afterwards until 8 h have passed. Absorbance values were registered for the entire available spectrum (from 200 to 1000 nm) and the absorbance maximum was found to be around 245 and 249 nm for budesonide and prednisolone, respectively.

2.7. Cell cultivation and viability assay

The EBC-1 human lung carcinoma cell line was used for cytotoxicity measurements. The cell culture medium was composed of Dulbecco's modified Eagle medium (DMEM, Thermo Scientific, USA) supplemented with the following ingredients: 10% foetal bovine serum (FBS, Thermo Scientific, USA), 1% penicillin-streptomycin mix (Thermo Scientific, USA), 1% L-glutamate (Thermo Scientific, USA), 1% sodium pyruvate (Thermo Scientific, USA), as well as 1% non-essential amino acids (NEAA, Thermo Scientific, USA). The cells were maintained under standard culture conditions (37 °C, 5% CO_2 and 100% humidity) and subcultivated at 70–80% confluence level using trypsin-EDTA (0.25%, Thermo Scientific, USA).

The cell viability tests were carried out in sterile white-walled optical bottom 96-well polystyrene plates (Thermo Scientific, USA). The EBC-1 cells were seeded at 20 000 cells/cm^2 concentration (6 200 cells/well) in 100 μl medium/well. After 24 h, when the cells already adhered to the plastic surface of the culture plates, the culture medium was removed and the cells were treated for 4 h with free corticosteroids or corticosteroid-containing 2C or 3C liposomes diluted with serum-free cell culture medium, similar to a previously published protocol [27]. Both prednisolone and budesonide were applied in five different concentrations (0.01, 0.1, 1, 10, and 100 μM) whereas liposomal suspensions in two concentrations (to reach 1 or 100 μM drug concentrations, respectively). In the case of the control group, no drugs or liposomes were added to the serum-free culture medium. After 4 h long treatments, the serum-free media were exchanged by the normal completed cell culture media (without any steroid or liposomes).

Cell viability was determined 24 h and 72 h later by applying the CellTiter Glo luminescent reagent (Promega, USA) according to the manufacturer's protocol. Luminescence was measured by means of a Fluoroskan FL Microplate Fluorometer and Luminometer (Thermo Scientific, USA) using the SkanIt software. The gained luminescence values are directly proportional to the cell number. In order to calculate the

relative cell numbers, all luminescence data were normalized to the mean value related to the untreated control measured after 24 h. Regarding each experimental group involving 6 parallel measurements, the relative cell number was expressed as the arithmetic mean ± standard error of the mean (SEM). For statistical analysis of the data, the Kruskal-Wallis nonparametric ANOVA was performed, followed by a median test applying the STATISTICA 10 software. A difference was considered statistically significant if $p < 0.05$.

2.8. Flow cytometry

In order to quantitatively examine the internalization of the different types of liposomes by the EBC-1 cells, fluorescence-activated cell sorting (FACS) analysis was carried out. For this purpose, the liposomes were fluorescently labelled by a PKH67 Green Fluorescent Cell Linker Mini Kit (Sigma-Aldrich, USA) according to the protocol described by Mousseau and co-workers [28]. Briefly, the commercial 1 mM ethanolic stock solution of the PKH67 fluorescent membrane dye was first diluted with ethanol until 0.1 mM concentration and then diluted further with PBS to 2 μM concentration. After that, this 2 μM PKH solution was mixed at 1:1 with the liposome suspension containing 2 g/L lipid (the original liposome samples with 10 g/L lipid content were diluted with PBS) and vortexed for 10 s. Finally, the so prepared suspensions of fluorescently labelled liposomes were maintained in the dark for 10 min and then diluted with serum-free cell culture medium to achieve 100 μM concentration regarding both corticosteroids, respectively.

The EBC-1 cells were seeded into 6 well plates at 94 000 cells/2 ml/well (10 000 cells/cm^2) and 24 h later their culture medium was replaced by the abovementioned suspensions of PKH-stained liposomes. According to the literature, the cellular uptake was assessed after 4 h long incubation [29] with the stained liposomes at 37 °C. For this purpose, the TrypLE^{TM} Express Enzyme solution (Thermo Scientific, USA) was applied after 4 h in order to dissociate the cells from the culture plates and to create single-cell suspensions. Subsequently, the cells were centrifuged (1200 rpm, 5 min), washed with PBS, pelleted (1200 rpm, 5 min) and resuspended in 400 μl PBS. After vortexing, the cell suspensions were analysed by applying a FACSCalibur flow cytometer (Becton-Dickinson, USA). The measurement results were evaluated by means of the CellQuest Pro software (Becton-Dickinson, USA) and the Flowing Software 2.5.1. (Turku Bioscience Centre, Finland). For each sample, ten thousand cells (gated events) were counted, and the fluorescent signal of the PKH67 dye was detected on the FL1 channel (Ex 488 nm; Em 530 nm). Untreated cells were also investigated as the negative control while PKH-treated cells were used as the positive control.

2.9. Microscopy studies

The growth of the EBC-1 cells during the viability investigations was followed by phase contrast microscopy applying an Axio Observer A1 inverted microscope (Zeiss, Germany) and photomicrographs were taken using the 10x objective and the AxioVision LE64 Rel.4.9.1. software.

In order to visualize the internalization of the fluorescently labelled liposomes, the EBC-1 cells were seeded into Nunc Lab-Tek Chamber Slides (Thermo Scientific, USA) with 8 wells at a density of 8000 cells/400 μl/well (10 000 cells/cm^2). After 24 h, the completed culture medium was replaced by PKH-solution or suspensions of PKH-stained liposomes, which were prepared and diluted with serum-free medium according to the same protocol as it was described at flow cytometry. Following a 24 h long incubation, the cells were washed with PBS and fixed by applying a 4% paraformaldehyde (PFA) solution (in PBS) for 20 min. After washing in PBS and rinsing with distilled water 3 times, respectively, the walls of the chambers were removed and the fixed samples were left to dry in the dark. Subsequently, the slides were mounted by means of the Prolong Gold antifade reagent (Molecular Probes, USA), which also contained a nuclear stain DAPI (4′,6-diamidino-2-phenylindole). The fixed specimens were stored in the dark at 4 °C until they were observed by confocal microscopy applying a Nikon Eclipse Ti2 Inverted Research Microscope (Nikon, Japan). The green fluorescence of the PKH dye was detected at 510 nm (Ex.: 488 nm), while the blue fluorescence of DAPI at 461 nm (Ex.: 405 nm). Using a 60x objective, images were taken with the help of the Abberior Imspector software (Abberior GmbH, Germany), and they were evaluated by the ImageJ program.

3. Results and discussion

3.1. Differential scanning calorimetry

Differential Scanning Calorimetry (DSC) measurements were carried out on six different types of liposome samples in order to determine their phase transition temperature in the physiologically relevant range ($T_{physiol}$). The samples varied in their membrane composition and the quality of the incorporated corticosteroid (Pred or Bud) if any was present. The T_m is a crucial parameter of drug-carrier liposomes as above this temperature, the membrane permeability is higher and the entrapped drug can be released easier [9]. This is affected by several variables, such as the presence of sterane framework compounds in the liposome [30].

The results of these measurements (Fig. 2.) were in accordance with the theoretical T_m values of liposomes composed of pure lipids and confirmed our previous calculations concerning the phase transition temperatures (around 39 °C) of the drug-free 2C- and 3C liposomes (Table 2.). The deviations of the measured T_m values from the theoretical ones regarding while for both 2C- and 3C liposomes were around 1.5 °C (Fig. 2.), which can be considered relatively small. These differences are supposedly due to the inaccuracy of the measurement (±0.5 °C) and the result of the interactions between the lipid and water molecules in the PBS-based liposome suspensions, which were not taken into account during theoretical calculations based on the molar ratio of the pure lipids.

Numerous experiments have investigated the effect of cholesterol on the T_m, which can break the steric structure of saturated fatty acid chains by wedging between the straight apolar chains or stabilize the membranes containing unsaturated fatty acids by fitting the gaps formed by the altered configurations due to the double bonds [31]. In our case, corticosteroid drugs with cholesterol-like structures play this role in the liposomes. Our DSC results (Fig. 2.) prove that the presence of a drug and its chemical quality does not affect the phase-transition temperature of the vesicles. After adding Pred or Bud, the deviation of the T_m value compared to the drug-free vesicles was only a maximum of 0.4 °C in the case of the 2C-liposomes (Fig. 2A) while for the 3C vesicles (Fig. 2B) it was only 0.3 °C. These changes provoked by the incorporation of the abovementioned steroid drugs into the lipid bilayers are considered negligible, keeping in mind the measurement errors of the DSC.

Similarly to our results, the T_m value of DPPC liposomes changed only by 0.7 °C after the incorporation of prednisolone [32]. Incorporating cholesterol in DPPC liposomes [33] or a corticosteroid drug, beclomethasone into DMPC liposomes led to only minor decreases in T_m values (the maximal deviation was 2 °C) [34]. It is noteworthy that the aforementioned vesicles are composed of only one kind of lipid component, whereas our liposomes are built up from at least two different phospholipids resulting in small gaps in the middle region of the bilayer formed by the shorter opposite phospholipid molecules. The relatively small corticosteroid molecules can wedge in these gaps and hence stabilize the membrane by preventing the flip-flop movement of the phospholipids. Consequently, this phenomenon can be explained that the usual destabilizing (and T_m decreasing) effect of steroid bonds [31] was not observed in the case of our liposomes.

The detailed analysis of the thermotropic parameters regarding the different types of liposomes can be seen in Table 3. According to the results the T-on-set and T-off-set, the peak of the phase transition is

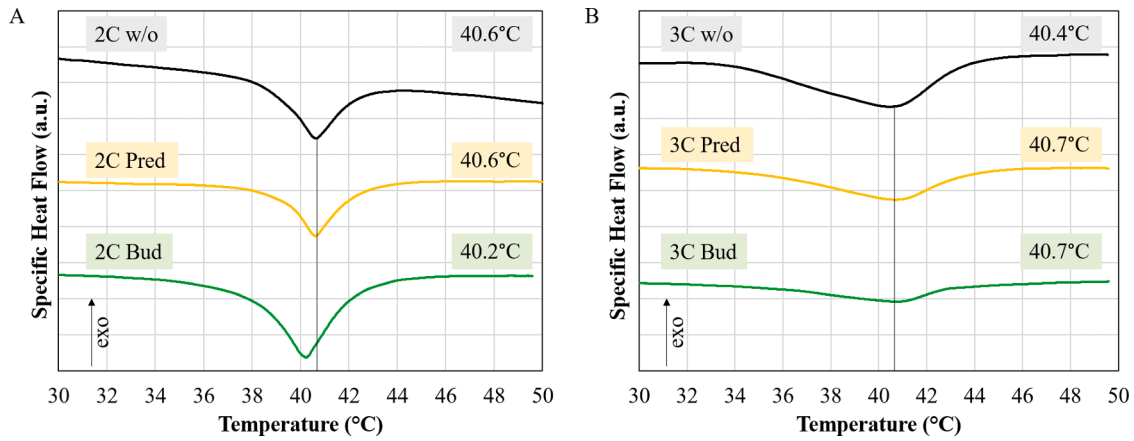

Fig. 2. Endothermic peaks determined by differential scanning calorimetry indicating the physiological phase transition temperatures regarding the 2-component (2C) liposomes (A) and 3-component (3C) liposomes (B) without drug (w/o), with prednisolone (Pred) and with budesonide (Bud). For comparison, the theoretical T_m values concerning 2C w/o and 3C w/o liposomes are 39.0 °C and 39.1 °C, respectively.

Table 3
Thermotropic features (T-on-set, T-off-set, $T_{physiol}$ and ΔH values) regarding 2-component (2C) and 3-component (3C) liposomes loaded with either prednisolone (Pred) or budesonide (Bud), or without drug (w/o). These data were determined by means of the TA Universal Analysis software according to the ISO 11357–3 international standard related to Differential scanning calorimetry.

Samples	2C w/o	2C Pred	2C Bud	3C w/o	3C Pred	3C Bud
T-on-set	38.9 °C	39.3 °C	38.1 °C	33.6 °C	35.0 °C	35.7 °C
T-off-set	42.5 °C	42.3 °C	42.4 °C	43.5 °C	43.6 °C	42.4 °C
$T_{physiol}$	40.6 °C	40.6 °C	40.2 °C	40.4 °C	40.7 °C	40.7 °C
ΔH	1.43 J/g	1.07 J/g	1.96 J/g	2.67 J/g	1.49 J/g	0.51 J/g

narrow (roughly ± 2 °C around the peak temperature) and symmetric in case of the 2C liposomes while it is much broader and asymmetric concerning the 3C liposomes. The incorporation of the corticosteroids did not influence the shape of the peak significantly. The enthalpy of the physiological phase transition was not significantly affected by adding drugs to the 2C liposomes. However, incorporation of Pred or Bud into the 3C liposomes led to a robust decrease in enthalpy, similarly to the literature data demonstrating lower enthalpies by increasing the cholesterol content of the liposomes [35]. This effect of cholesterol is explained by its rigid structure which can embarrass the movement of the phospholipid molecules. It is noteworthy, that in case of some corticosteroid drugs (beclometasone dipropionate) was found to increase the phase transition enthalpy of DMPC liposomes [34]. Consequently, the corticosteroid drugs may have another effect on the phase transition of the liposomes as the cholesterol and their effect is influenced by the lipid composition of the liposomes.

3.2. *Long-term (storage) stability of the liposomes*

The liposome samples with different compositions were stored at room temperature in PBS for 6 months and their size distribution was measured regularly by dynamic light scattering: weekly in the first month, then monthly for half a year (Fig. S6.). We can conclude that the size distribution regarding both Pred and Bud-containing liposomes was considerably narrow showing a peak with a maximum of around 120 nm even after 6 months,the average polydispersity index (PDI) was between 18.9 and 21.0%. However, liposomes without any drug tended to form aggregations or fused into large vesicles in the undesired size range (above 1 μm in diameter). This phenomenon was remarkable in the case of empty 2C liposomes after 3 months. Moreover, we could also detect some aggregations concerning Pred-loaded 3C liposomes although in a much smaller amount (Fig. S6.).

Our results show that the hydrodynamic diameter (based on the Z-average) remained in a narrow range (approximately between 120 and 140 nm, with average PDI values of 20.0% and 19.0%, respectively) in the case of both prednisolone- and budesonide-loaded 2C liposomes (Fig. 3.). However, the 2C liposomes without any drug proved to be more unstable having more than 180 nm average diameter after 5 months and indicating signs of aggregation after 6 months (average PDI was 24.0%). Contrary to this, the 3C liposomes did not demonstrate any remarkable changes in their hydrodynamic diameter throughout this time period regardless of their membrane composition. The average diameter of 3C samples containing Pred or Bud and even without any drug remain in a narrow range (between 120 and 150 nm, with average PDI of 21.0% and 18.9%) for half a year. It seems that the chemical quality of the corticosteroid does not affect the stability of the vesicles but the lipid composition of the membrane plays a more important role in this aspect.

The precise values of the average hydrodynamic diameter and PDI after 3- or 6-month-long storage are summarized in Table 4. We can observe that the average hydrodynamic diameter values concerning 5 of the 6 different liposome formulations at each experimental time point were in the range between 120 and 150 nm. The exception was the group of the 2C liposomes without drugs showing a very high average hydrodynamic diameter value (560.4 nm), which indicates a strong effect of aggregation or fusion in the case of this liposomal formulation. For this type of liposome was determined the highest PDI value (22.6% after 3 months and 24.0% after 6 months), which also suggests that the particle size in these liposome suspensions was not homogenous as it can be observed on the diagram (Fig. S6). In all other cases and time points, the calculated PDI values were found to be between 8.1% and 21.0%, confirming the narrow and practically unimodal particle size distributions (Fig. S6).

Therefore, the 2C liposomes without any drug were the least stable and, in that case, aggregation occurred after 3 months. However, no remarkable aggregation was observed in the case of the 4 types of drug-containing liposomes and regarding the 3C liposomes without drug.

Although the membrane composition seems to be a deterministic factor in terms of long-term stability, similar results were described in the case of liposomes composed of a single kind of phospholipid (DMPC, DPPC or DSPC) [35]. Briuglia et al. followed the size distribution of the vesicles stored in PBS for 30 days and they measured maximum ± 10% changes in the hydrodynamic diameter while most stabile liposomes were created by adding cholesterol. Our liposomes consist of the same lipids but in combination as well as they contain no cholesterol. However, as we stated earlier, our drugs (being synthetic derivatives of steroid hormones) have very similar chemical structures and due to this fact, they can act in the same way since they are also integrated in the

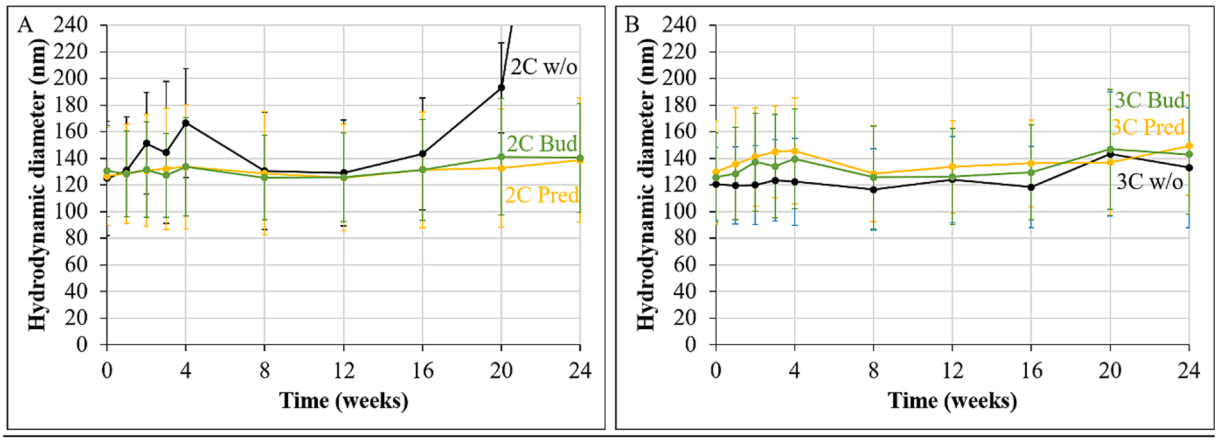

Fig. 3. . Long-term stability of the liposomes followed by dynamic light scattering. Changes in the hydrodynamic diameter during the first 6 months after preparation of the two-component (2C) liposomes (A) and three-component liposomes (B) without a drug (w/o), with prednisolone (Pred) and with budesonide (Bud).

Table 4
Changes in hydrodynamic diameter (D_H) and polydispersity index (PDI) during long-term storage of liposomes in PBS: immediately after preparing (0 month – 0 m), as well as 3 months (3 m) and 6 months (6 m) later.

Samples		2C w/o	3C w/o	2C Pred	3C Pred	2C Bud	3C Bud
0 m	D_H (nm)	125.0 ± 27.4	120.7 ± 27.3	126.6 ± 36.6	126.6 ± 38.9	130.4 ± 34.5	125.6 ± 33.4
	PDI (%)	17.6 ± 6.3	8.7 ± 2.5	13.3 ± 2.2	14.8 ± 6.1	12.9 ± 7.6	14.7 ± 6.0
3 m	D_H (nm)	129.2 ± 32.6	124.0 ± 32.4	125.5 ± 39.9	133.7 ± 34.9	125.7 ± 33.3	126.3 ± 35.9
	PDI (%)	22.6 ± 3.0	12.3 ± 2.8	16.3 ± 3.1	15.8 ± 7.4	14.5 ± 1.4	15.2 ± 5.4
6 m	D_H (nm)	560.4 ± 28.8	133.0 ± 45.0	138.6 ± 46.7	149.5 ± 37.4	140.3 ± 40.9	143.0 ± 44.8
	PDI (%)	24.0 ± 2.5	19.9 ± 0.6	20.0 ± 3.5	21.0 ± 5.4	19.0 ± 0.9	18.9 ± 1.8

membrane. In addition, competition can occur in the phospholipid bilayer between cholesterol and hydrophobic drugs leading to lower entrapment efficacy of the drug [36]. Therefore, it would be disadvantageous in our case to build cholesterol in the liposomes.

Nevertheless, such long-term (for several months) stability tests as ours are exceptionally rare in the literature, especially with corticosteroid-loaded liposomes. In conclusion, our data show that all the Pred or Bud-containing liposome formulations (both 2C and 3C liposomes) can be stored at room temperature in the form of aqueous suspension (in PBS) for a minimum of half a year without a decrease in their stability.

Before the investigation of the internalization of the liposomes by the cells, the PKH67 fluorescent dye was incorporated into the lipid bilayer of the liposomes. The possible changes in the size distribution of our liposome samples after incorporating PKH67 dye were investigated by dynamic light scattering (Fig. S7.). The results proved that the presence of the PKH67 molecules in the lipid bilayer had no significant effect on the size distribution of the liposome samples in either case.

Before adding the liposomal suspensions to the cells, we studied the changes in the average hydrodynamic diameter of the liposomes when their environment was the cell culture medium (MEM) instead of the PBS buffer used for the storage (Table 5). According to our results, the physiological conditions (provided by MEM) only slightly influenced the liposome size showing merely a 1.7–8.6% (2–10 nm) increase in the average diameter. However, the average hydrodynamic diameter of the different kinds of liposomes remained in the range between 120 and 126 nm also in MEM after 4 h.

During this stability test, we incubated our liposomes in the cell culture medium (MEM) for 4 h, similarly to the later performed cell viability and internalization assays. Other studies followed the size distribution and the hydrodynamic diameter of the liposomes under *in vitro* conditions also for a few hours or a maximum of one day. Hypericin and cyclodextrin-hypericin-loaded DPPC and DSPC liposomes with a bit larger size (200–300 nm in diameter) than ours were investigated in IMDM medium with 10% FBS for 24 h. Regarding the DSPC-hypericin liposomes, the diameter grew by 15–20% in 1 h but their size dropped back to almost the original size after 24 h. The size of the DPPC-based liposomes changed just slightly after 1 h, but after 24 h their diameter increased by 20–50% [37]. Our liposomes are composed predominantly of DPPC, so the aforementioned data are in accordance with our results showing only a minor increase in liposome size during a few hour-long-incubation in cell culture medium.

We can conclude that all liposome types have a slightly negative zeta potential value (Table 6), which is affected both by the phospholipid

Table 5
Comparison of hydrodynamic diameter (D_H) and polydispersity index (PDI) regarding liposomes with different compositions measured in PBS and in MEM after 4 h-long incubation.

Samples		2C Pred	3C Pred	2C Bud	3C Bud
PBS	D_H (nm)	112.0 ± 28.7	117.9 ± 28.2	115.8 ± 24.4	119.9 ± 33.6
	PDI (%)	11.0 ± 3.0	12.1 ± 5.2	4.1 ± 1.0	18.8 ± 4.1
MEM	D_H (nm)	120.8 ± 44.1	124.9 ± 39.1	126.0 ± 44.3	121.7 ± 39.2
	PDI (%)	23.0 ± 0.3	14.3 ± 7.4	19.3 ± 2.7	22.2 ± 1.1

Table 6
Characterization of the zeta potential values (mean zeta potential and standard deviation) regarding 2-component (2C) and 3-component (3C) liposomes loaded with either prednisolone (Pred) or budesonide (Bud), or without drug (w/o). These data were determined by the Anton Paar Litesizer particle analyzer software Kalliope.

Samples	2C w/o	2C Pred	2C Bud	3C w/o	3C Pred	3C Bud
Zeta potential (mV)	−7.26 ± 2.05	−6.93 ± 0.82	−3.64 ± 0.44	−1.81 ± 0.55	−7.78 ± 0.49	−10.84 ± 0.23

composition and the entrapped drug itself. Concerning the two-component liposomes, the drugs seem to reduce the negative charge of the liposomes. However, in case of the three-component liposomes we can see enhanced charges after adding Pred or Bud. All of the measured zeta potential values in our systems were between −1 and −11 mV, in line with the literature data regarding zeta potential ranges of DPPC liposomes [38] and liposomes composed of DPPC-DMPC mixtures [25].

3.3. Attenuated total reflectance (ATR) Fourier-transform infrared spectroscopy (FTIR)

In order to characterize the interactions between the liposomal membrane and the incorporated drug molecules, ATR-FTIR measurements were performed. The spectra of the three membrane-forming phospholipids (DPPC, DSPC, DMPC) and the two applied drugs (Pred and Bud) as well as the 6 different types of liposomes are represented in Fig. 4.

Due to the very similar chemical structure of the three phospholipids, the characteristic peaks can be observed on all the three spectra (Fig. 4A and B), namely two peaks between 2850 and 2920 cm^{-1} corresponding to the symmetric and asymmetric stretching of the methylene ($–CH_2$) groups, which are parts of the fatty acids in lecithin, a peak at 1735 cm^{-1} related to the stretching vibration of the carbonyl (-C = O) group in the ester bond as well as the peak at 1090 cm^{-1} indicating the symmetric stretching of the $-PO_2$ group [39–41].

Considering the structural similarities of the two corticosteroid drugs, identical peaks are recognisable in their spectra (Fig. 4A and B) in the whole range, e.g. the two peaks between 1650 and 1740 cm^{-1} correspond to the stretching vibrations of the acetyl -C = O groups and the dihydrobezoquinone -C = O groups while at 3666 cm^{-1} the characteristic vibration peak of the OH-group can be seen [42,43].

Regarding the spectra of the liposomes (Fig. 4C and D), the big peak of –OH stretching related to water are conspicuous between 3000 and 3600 cm^{-1}. The peaks corresponding to the $–CH_2$ groups of fatty acids

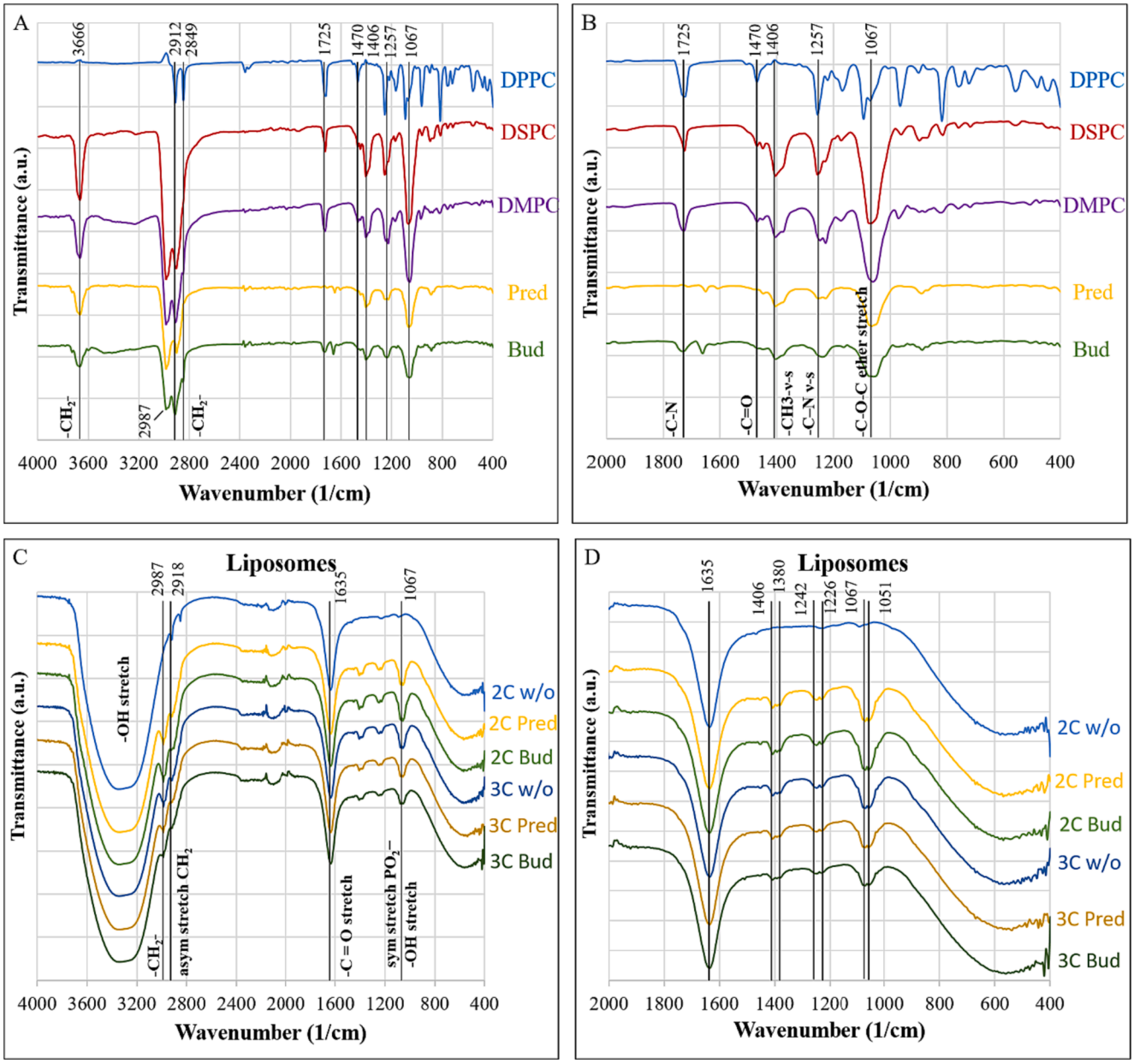

Fig. 4. FTIR spectra regarding the different components in powder form (A & B) as well as the two-component (2C) or three-component (3C) liposomes containing Pred or Bud or without a drug (w/o) (C & D), demonstrating the whole wavenumber range (A & C) and the enlarged fingerprint region.

can be seen at 2987 and 2918 cm^{-1} while the peaks related to the -C = O stretching can be observed at 2918 cm^{-1}. Concerning -PO_2 groups, the peaks at 1226 cm^{-1} and between 1046 and 1067 cm^{-1} represent the asymmetric and symmetric stretching, respectively ([44–47]). Comparing the spectra of the empty and the drug-containing liposomes (Fig. 4C and D), identical regions can be discovered (several peaks between 1057 and 1407 cm^{-1}). This indicates that the presence of the steroid drugs did not influence the structure of the phospholipid membranes significantly. Therefore, only weak physical interactions occur during encapsulation of the corticosteroids into the phospholipid membrane, which ensures that the biological efficacy of the drugs is not decreased by entrapment.

3.4. Drug entrapment capacity and drug leakage

The entrapment efficiencies of Pred and Bud regarding both liposomal compositions were determined by means of size-exclusion gel chromatography. In the beginning, the liposome fractions containing only entrapped drugs could be collected. However, the free drug (that is not entrapped i.e. is excluded) can be found only in the later fractions, because these small molecules require longer elution time (Fig. S3). According to our results (Table 7), more than 70% was entrapped from both corticosteroids in the case of both liposomal compositions. These high values can be explained by the highly lipophilic character of the corticosteroids.

The entrapment efficiency related to the PKH67 fluorescent membrane dye, which was used for the cellular internalization studies for labelling of the liposomes, was checked by applying high-performance liquid chromatography combined with fluorescence detecting and we found that more than 94% of the PKH dye was successfully incorporated into the liposomes (Table 7).

Kaddah et al. have investigated several commonly used corticosteroids in DPPC liposomes and they measured really high encapsulation efficiency values. For prednisolone, it was 97.25% but in the case of the other drugs it also exceeded 94% [48], so these results correspond with our data (Table 5). Although they did not examine budesonide, our results concerning the entrapment efficiency of this drug also fit in this line due to the similarities of its chemical structure to the other corticosteroids.

Previous studies implied that hydrophobic drugs, which have a higher affinity towards the lipid bilayer, compete with cholesterol if it is present in the membrane [49]. So, in our case, the lack of cholesterol contributed to the high entrapment efficiency of Pred and Bud. Furthermore, due to the fact that we used the lipophilic form of the corticosteroids instead of their more water-soluble salt forms (which is often used according to the literature), we utilized a simple physicochemical law to ensure as high entrapment efficiency as possible.

In the future, the intended administration route of our liposomes would be inhalation. They could supposedly fusion in a short time with the surfactant layer of lung alveoli (having DPPC as the major lipid component) containing 90 (w/w) % lipids and 31% of this lipid content is composed of DPPC [22], which is at the same time the main lipid component of the liposomes of this study. Therefore, drug leakage would not be really relevant considering therapeutic application but it can be interesting in respect of storage. Consequently, we followed the drug leakage from the liposomes by dialysis (against PBS) for each type of corticosteroid-loaded liposomes for 4 h a bit under and above the physiological phase transition temperatures (~39 °C) but still in the range possibly occurring in the living human body (Fig. S8.).

Table 7
The entrapment efficiencies for Pred and Bud determined by size-exclusion chromatography (SEC) followed by UV–Vis spectroscopy as well as for PKH67 fluorescent dye determined by high-performance liquid chromatography (HPLC) applying a fluorescence detector.

Liposome	Method	Exclusion (%)	Entrapment (%)
2C Pred	SEC	29.46	70.54
3C Pred	SEC	27.46	72.54
2C Bud	SEC	13.25	86.75
3C Bud	SEC	13.30	86.70
PKH67	HPLC	5.15	94.85

No major differences could be observed in the drug leakage between the normal body core temperature (37 °C) and a bit above the temperature of the human tissues during strong inflammation (41 °C). The 4 h-long incubation provides information about the changes under the condition during the *in vitro* studies. However, considering that the potentially inhaled drugs do not stay on the surface of the alveoli for hours, the leakage of corticosteroids within the first 30 min is more relevant in respect of the future possible human application. By this time, the drug leakage was 35–40% for Pred and 6–8% for Bud. The leakage of Pred-release started from a higher ratio after 10 min and reached its plateau after 60–90 min at around 55% at both temperatures independently from the membrane composition of the vesicles, corresponding to 3.5% of the solubility limit (223 mg/L in water). In contrast, the Bud-loaded liposomes were able to retain a much higher ratio of the incorporated drug, and the maximum released drug ratio remains between 15 and 20% for both samples at both temperatures even after 4 h. The remarkably low level of Bud release might be the consequence of the extremely low water solubility (10.7 mg/L in water) of this drug. Consequently, the more than 20fold higher water solubility of Pred is in accordance with the elevated leakage compared to Bud. Therefore, the entrapment of Bud is more effective in the case of our liposome formulations.

Hosseini et al. developed phosphatidylcholine vesicles with an average size of 186 nm for intramuscular administration for the delivery of Pred-acetate. They examined drug release by dialysis in PBS buffer through several days and the results showed a zero-order process of drug-leakage from the liposomes. These liposome samples were able to retain approximately 80% of the encapsulated Pred-acetate even after roughly 120 h [50].

3.5. Cell viability test

Moreover, budesonide may have a remarkable role in the future during the battle against COVID-19 since its antiviral activity against SARS-CoV-2 was proved by *in vitro* tests [20] whereas a clinical trial demonstrated that inhalation of budesonide in the early phase of infection can reduce the risk of hospitalization [21].

The cell viability assay aimed to determine how the abovementioned two corticosteroids and their liposomal formulations influence the vitality and proliferation of the EBC-1 human lung carcinoma cells. This cell line was chosen because of that our future plan is to develop an inhalable liposomal drug for possible asthma therapy. According to our results concerning the effect of the free Pred (Fig. 5A) and Bud (Fig. 5C), these commonly used drugs turned out as non-toxic compounds for EBC-1 cells, as expected. Interestingly, lower concentrations of Pred (0.1 and 1 μM) slightly reduced the relative cell number compared to that of the untreated control (Fig. 5A). In addition, 0.1 μM or 100 μM of Bud also led to decreased cell number after 24 h but they reached the control level after 72 h (Fig. 5C). However, both Pred (Fig. 5A) and Bud (Fig. 5C) permitted cell proliferation from 24 h to 72 h in case of each concentration investigated. Their liposomal forms (Fig. 5B for Pred and 5D for Bud) neither show a drastic effect on the vitality of these cells. We detected minor, but significant changes in the relative cell number compared to control for certain groups after 24 or 72 h (e.g. in the case of 2C and 3C liposomes loaded with 100 μM Pred or Bud). We can still conclude that liposomal drugs can also be considered non-toxic for these cells since neither empty nor drug-loaded liposomes prevented the proliferation of the EBC-1 cells from 24 h to 72 h (Fig. 5B and 5D). The phase contrast microscopy study (Fig. S9.) confirmed the measured viability data, showing much more cells at 24 h (Fig. S9A) than at 72 h (Fig. S9B).

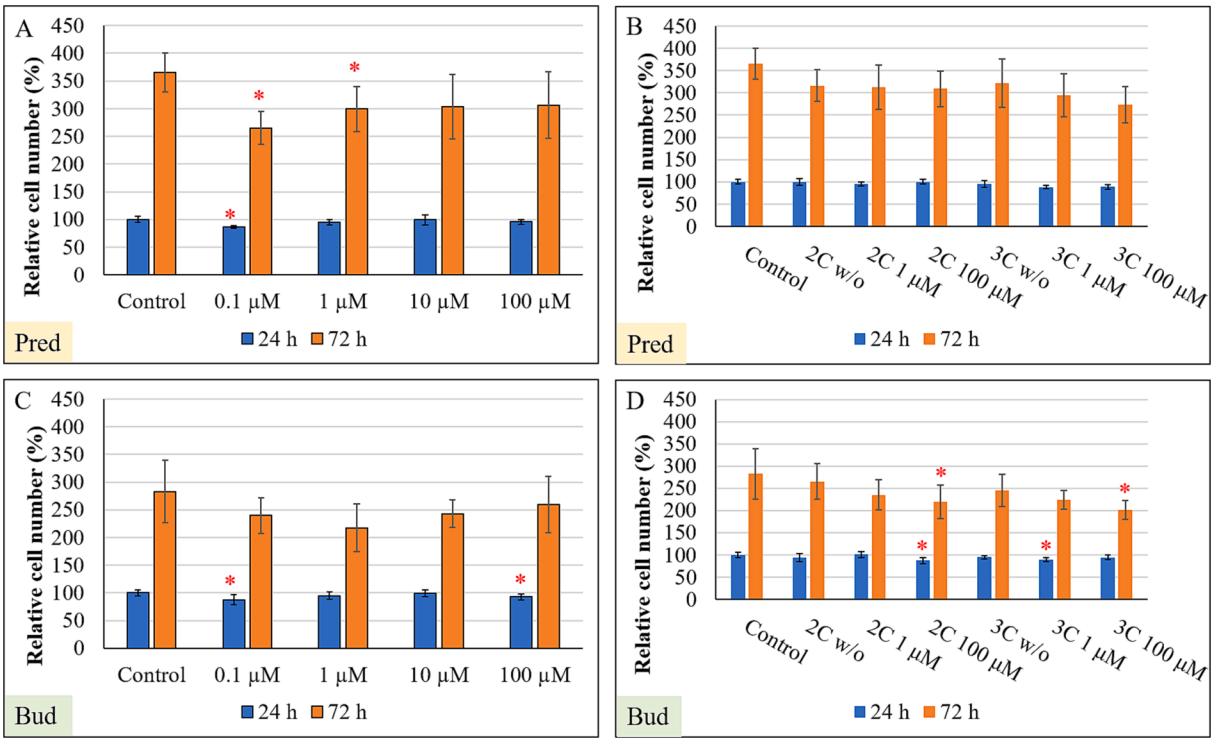

Fig. 5. Results of the viability assay performed on EBC-1 cells 24 h and 72 h after the treatments with prednisolone or budesonide *per se* in different concentrations (A & C) as well as with two-component (2C) or three-component (3C) liposomes containing Pred or Bud or without a drug (w/o) (B & D). The untreated cells were used as a control. In the case of liposomes, the concentration of both drugs was set to either 1 μM (2C 1 or 3C 1) or 100 μM (2C 100 or 3C 100) in each well. The relative cell number data were determined by normalization of the measured absorbance values to the values of control at 24 h. Significant changes ($p < 0.05$) in relative cell numbers compared to the relevant control groups are labelled with an asterisk (*).

Comparing the effect of free (Fig. 5A) and liposomal form of Pred (Fig. 5B) at the same concentration, no remarkable differences can be observed in the case of 2C vesicles whereas incorporation of Pred into 3C liposomes slightly decreased the relative cell number. Nevertheless, treatment with both free (Fig. 5C) and liposomal Bud (Fig. 5D) at 100 μM concentration resulted in reduced cell viability (by 25–30%) at certain time points.

According to our *in vitro* results (Fig. 5.), we can conclude that neither prednisolone nor budesonide *per se* is able to inhibit the growth of the EBC-1 human lung carcinoma cells. Similarly, 200 μM Pred did not influence the viability of pulmonary artery smooth muscle cells [51]. Regarding melanoma and colon carcinoma cell lines, Banciu et al. found a 10–20% decrease in cell viability after treatment with empty liposomes composed of DPPC, cholesterol and PEG2000-DSPE [52], which is in accordance with our data regarding liposomes without drugs. Applying the aforementioned liposome compositions, 72 h-long treatment with Pred-loaded liposomes resulted in only moderately reduced cell viability (similarly to our data) while in the case of Bud-loaded liposome (Bud-concentration: 100 μM) the reduction in cell viability was much higher (70%) [52] than in our case (25–30%, Fig. 5).

3.6. Internalization of the liposomes

In order to quantify the cellular uptake of the liposomes, flow cytometry was performed (Fig. 6.) The flow cytometric measurements prove that the PKH67-labelled liposomes could be internalized by the EBC-1 cells (Fig. 6.). The untreated control showed only a low basal level of fluorescence, which can be explained by the autofluorescent property

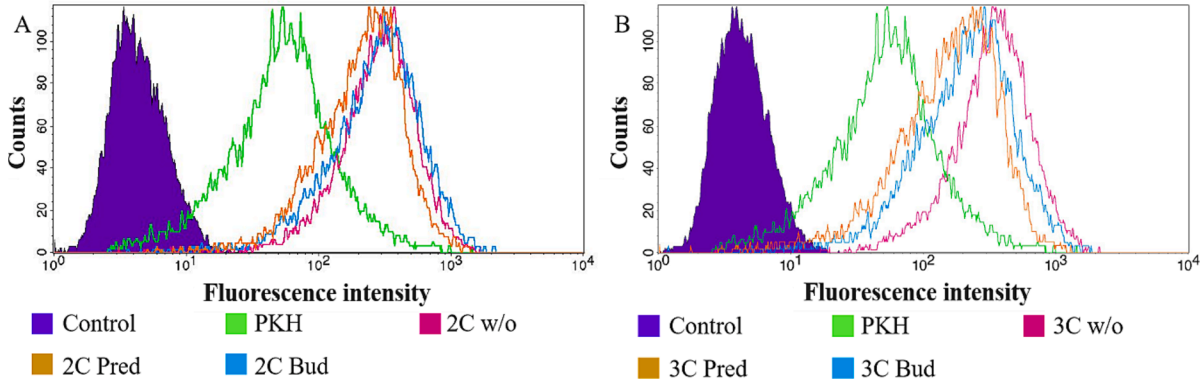

Fig. 6. Flow cytometric analysis concerning the uptake of fluorescently labelled two-component (2C) liposomes (A) and three-component (3C) liposomes (B) by EBC-1 cells. Untreated cells were regarded as negative control (Control) whereas cells treated with solutions of the PKH67 fluorescent dye served as positive control (PKH group). Liposomes containing prednisolone (Pred), budesonide (Bud) or no drug (w/o) were also investigated.

of all cells due to the aromatic amino acids in various proteins or the nucleotides. In the case of the cells treated with PKH67 solution (PKH-control) much higher fluorescence intensity values were measured. In addition, the fluorescence intensities after treatment with different kinds of PKH-labelled liposomes were found to be one order of magnitude higher compared to the PKH-control. Therefore, the liposomes labelled with PKH67 could be taken up by the cells in a much higher amount (approximately tenfold in quantity) than the PKH67 molecule alone. According to our results, the chemical composition of the liposomes (2 or 3 lipid components, with or without corticosteroid) does not affect the uptake ratio.

Consequently, the flow cytometric analysis revealed that all 6 different liposomal formulations can enter the EBC-1 cells.

The internalized PKH-labelled liposomes were visualized by confocal microscopy (Fig. 7.). The fluorescent images show that strong bright green dots (corresponding to the PKH-67-labelled liposomes) can be found in the whole cytoplasm of the EBC-1 cells regardless of the lipid composition (2C or 3C) or the incorporated drug (Pred or Bud; Fig. 7.). This measurement proves that all kinds of liposomes get inside the cells in high amounts and corresponds to the FACS results. Although PKH-leakage of the vesicles is possible, lots of tiny spots can be distinguished. The pale homogenous staining in the cytoplasm of the untreated control group can be considered merely background fluorescence originating from cytoplasmic proteins. Regarding the treatment with PKH67-solution (PKH control), the bigger green spots all over the cells supposedly represent small droplets of the dye solution which remained on the samples after fixation of the cells.

In the internalization of liposomes by the cells, several processes are involved like adsorption, membrane fusion or endocytosis. The interactions between the liposomes and the cell membrane highly depend on the structure and lipid composition [53]. Certain lipids such as cholesterol have an important role in membrane fusion [54]. We suppose that in our case, the main mechanism regarding the internalization of the liposomes is also the membrane fusion and instead of cholesterol, the entrapped corticosteroids may be also involved in this process.

In summary, the results of the confocal microscopy study confirmed the finding of the flow cytometric analysis, namely that both 2C and 3C liposomes with or without drug can be taken up by the EBC-1 cells.

4. Conclusion

We successfully prepared prednisolone and budesonide-containing liposomes (with an average diameter of around 100 nm) composed of 2 or 3 phospholipids (DPPC, DMPC and DSPC), which can be stored in PBS at room temperature for at least half a year without signs of aggregations. The lipid composition was set to reach the main transition temperature typical for the inflamed tissues (being the highest drug release around this temperature) and the data measured by DSC confirmed our previous calculations. The presence of corticosteroid drugs did not influence the main transition temperature. The hydrodynamic diameter remained in the desired range throughout 6 months regarding all drug-containing liposomes. All of the liposome types have a slightly negative zeta potential value. The Fourier-transform infrared spectroscopy revealed no chemical interaction between the drug and lipid molecules. The entrapment efficiency was between 70 and 87% in the case of both drugs whereas the leakage of the Pred was higher due to its higher water solubility. None of the corticosteroid-loaded liposome types is toxic for the EBC-1 human lung carcinoma cell line and all of them can be internalized by the cells according to both confocal microscopy and flow cytometry.

In conclusion, the developed liposomes could be useful in the delivery of corticosteroids (Pred or particularly Bud) in more effective

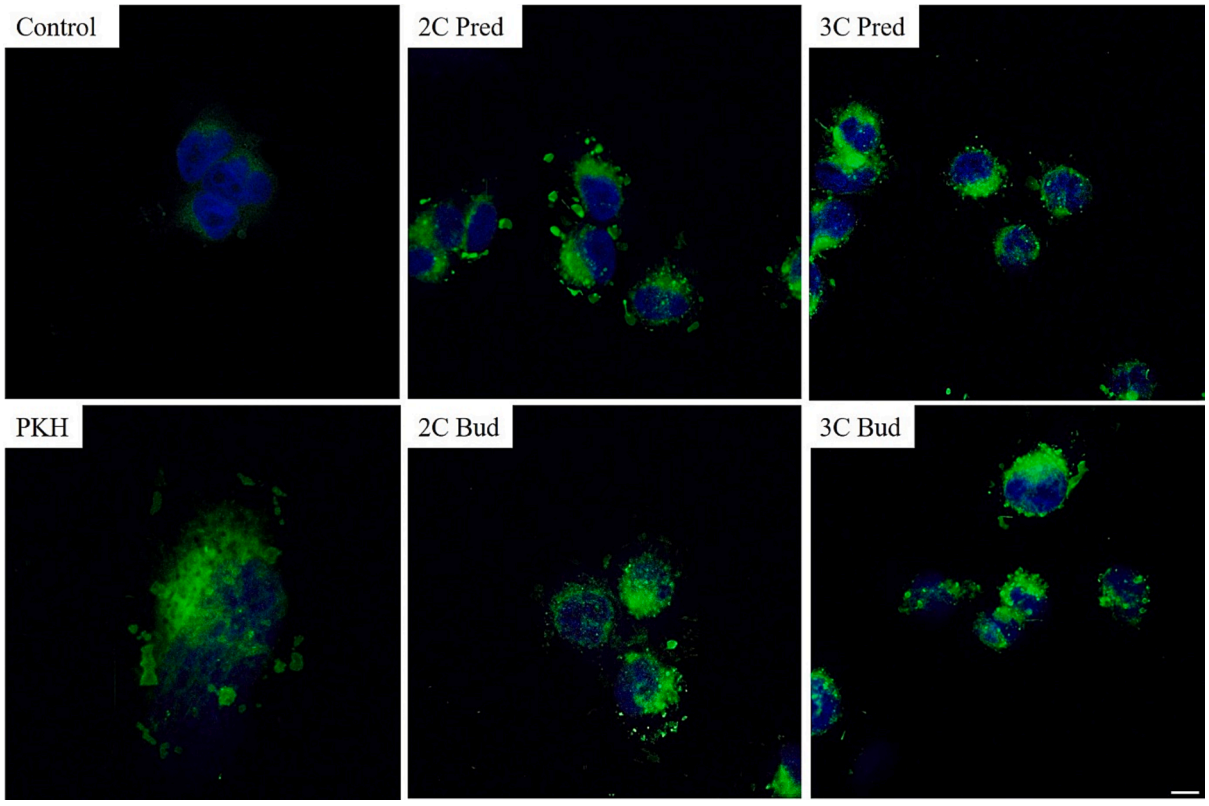

Fig. 7. Internalization of PKH67 fluorescent dye (green) labelled liposomes by EBC-1 cells. The cell nuclei were stained with DAPI (blue). Untreated cells were regarded as the negative control (Control) whereas cells treated with solutions of the PKH67 fluorescent dye served as the positive control (PKH group). Two-component (2C) and three-component (3C) liposomes containing prednisolone (Pred) or budesonide (Bud) were also investigated. The images were taken by confocal microscopy at the same magnification. The scale bar indicates 10 μm.

asthma therapy, having fewer side effects due to the nanoformulation. However, further investigations are needed including in vivo experiments before application in human clinical trials.

CRediT authorship contribution statement

Bálint Budavári: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft. **Áron Karancsi:** Data curation, Investigation, Writing – original draft. **Balázs Gábor Pinke:** Data curation, Formal analysis, Investigation. **Éva Pállinger:** Formal analysis, Investigation, Methodology. **Krisztina Juriga-Tóth:** Investigation, Visualization. **Márton Király:** Data curation, Investigation, Methodology. **Zsófia Szász:** Investigation, Visualization. **István Voszka:** Methodology, Supervision. **Kolos Molnár:** Funding acquisition, Supervision, Writing – review & editing. **László Kőhidai:** Resources, Supervision. **Angela Jedlovszky-Hajdu:** Conceptualization, Funding acquisition, Formal analysis, Project administration, Supervision, Writing – review & editing. **Krisztina S. Nagy:** Conceptualization, Formal analysis, Supervision, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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